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1)**Enzyme therapy for Pompe disease: From science to industrial enterprise. .** European Journal of Pediatrics, (October, 2002) Vol. 161, No. Supplement 1, pp. S106-S111. print. ISSN: 0340-6199.

2)**Enzyme therapy for Pompe disease with recombinant human a-glucosidase from rabbit milk**
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3) **Expression of cDNA-encoded human acid alpha-glucosidase in milk of transgenic mice. .** BIOCHIMICA ET BIOPHYSICA ACTA, (1996 Aug 14) 1308 (2) 93-6.

Thank you

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Enzyme therapy for Pompe disease with recombinant human α-glucosidase from rabbit milk

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Summary: Pompe disease is a metabolic myopathy caused by deficiency of lysosomal acid α-glucosidase. In this report we review the first 36 weeks of a clinical study on the safety and efficacy of enzyme therapy aimed at correcting the deficiency. Four patients with infantile Pompe disease were enrolled. They received recombinant human α-glucosidase from transgenic rabbit milk. The product is generally well tolerated and reaches the primary target tissues. Normalization of α-glucosidase activity in skeletal muscle was obtained and degradation of PAS-positive material was seen in tissue sections. The clinical condition of all patients improved. The effect on heart was most significant, with an impressive reduction of the left ventricular mass index (LVMI). Motor function improved. The positive preliminary results stimulate continuation and extension of efforts towards the realization of enzyme therapy for Pompe disease.

Pompe disease is a lysosomal storage disorder involving heart and skeletal muscles (Hirschhorn and Reuser 2000). The most serious form of the disease is seen in babies with the infantile form of the disease. Feeding difficulties, respiratory infections, and motor delay are the presenting symptoms in the first months of life. A characteristic increased heart size, due to the presence of a hypertrophic cardiomyopathy, is seen on chest radiographs in all patients, and often raises suspicion of the diagnosis. Both

cardiac failure and respiratory insufficiency add to the cause of death. Patients usually do not survive beyond the age of one year.

A milder course is seen in patients with late-onset forms of the disease. Symptoms may start at any age and are related to progressive dysfunction of skeletal muscles. The heart is mostly not involved. When the disease proceeds, patients become wheelchair bound and dependent on artificial ventilation. A scoliosis may develop. Respiratory failure is the main cause of death. The age of death usually depends on the rate of progression of the disease and degree of involvement of respiratory muscles; it varies from early childhood to late adulthood.

The disease is caused by a deficiency of acid α-glucosidase needed for degradation of lysosomal glycogen (Hers 1963). There is generally good correlation between the level of residual enzyme activity and the severity of disease (Reuser et al 1995). α-Glucosidase activity is virtually absent in the severe infantile cases, whereas residual activities up to 20% of normal are seen in late-onset patients.

The rationale of enzyme therapy for treatment of lysosomal storage disorders is based on the natural process whereby extracellular compounds gain access to the lysosomal system via endocytosis. The first and subsequent attempts at enzyme therapy for Pompe disease date from 1964, but these failed (Baudhuin et al 1964; Hug and Schubert 1967; De Barsy et al 1973). α-Glucosidases from nonhuman sources appeared antigenic and low doses were administered compared to the dose we presently think is required to correct the deficiency in skeletal muscles. Developments in DNA technology have given enzyme therapy for lysosomal storage diseases a new chance by allowing large-scale production of recombinant human enzymes (Grabowski et al 1995; Bijvoet et al 1998). The present report covers the first 36 weeks of a study on enzyme therapy in patients with infantile Pompe disease with recombinant human α-glucosidase produced in the milk of transgenic rabbits.

METHODS

Study design: The clinical study was performed at the Sophia Children's Hospital, Rotterdam, The Netherlands, and was a single-centre, open-label pilot study. The study was approved by the Institutional Review Board. Written informed consent was obtained from the parents. All assessments were performed at baseline and on regular basis thereafter. Four patients with infantile Pompe disease were included.

Muscle biopsies were scheduled at baseline and subsequently at 12-week intervals to assess uptake of α-glucosidase and changes in histopathology. Biopsies were taken from the quadriceps muscle via an open muscle biopsy, one day after infusion of recombinant human α-glucosidase (rabbit milk rhAGLU).

Neuromotor and mental development were assessed with the Alberta Infant Motor Scale (AIMS) every 4–6 weeks (Piper and Darah 1994), the Bailey Scales of Infant Development (BSID II) every 12 weeks (Bailey 1993), and regular standardized neurological examinations.

Biochemistry: Tissue specimens for measurement of α-glucosidase activities were immediately frozen in liquid nitrogen and stored at −80°C until use. The tissue was homogenized in water. α-Glucosidase activity was determined using the artificial substrate 4-methylumbelliferyl (MU)-α-glucopyranoside at pH 4.0 (Reuser et al 1978). Protein concentration of the supernatant was determined using the bicinchoninic acid (BCA) protein assay as described by the manufacturer (Pierce, USA).

Histology: For histology purposes, tissue specimens were fixed in 4% glutaraldehyde and embedded in glycol–methacrylate (GMA). Tissue sections (4 μm) were stained with periodic acid–Schiff (PAS) reagent. Slides prepared at different time points were stained in one session.

Enzyme production: rhAGLU production in transgenic rabbits was achieved essentially as previously described (Bijvoet et al 1999). An overview of the procedure is given in the Results section. The milk is defatted, caseins are removed by filtration, and rhAGLU is purified by multistep column chromatography. Additional measures are taken to remove hazardous contaminants, for instance viruses that can potentially be present. Both breeding of rabbits and downstream processing of rabbit milk rhAGLU were performed by Pharming N.V., Leiden, The Netherlands.

RESULTS

Production of recombinant human α-glucosidase (rhAGLU): The recombinant α-glucosidase that was used in the study is produced in transgenic rabbits by the epithelial cells of the mammary gland. The gene construct used for this purpose encompasses the human α-glucosidase gene with all exons and introns, including untranslated exon 1 (28.5 kb) and 9 kb of 3′-UTR. High-level, cell type-specific expression is obtained by fusing this gene at the 5′ side to 6.3 kb of the bovine α_{S1}-casein promoter. Figure 1 depicts the transgene and illustrates the further procedures. The transgene is microinjected into the pronucleus of fertilized rabbit oocytes. These are implanted in foster mothers. Newborns carrying the transgene are selected by Southern blot analyses; those that transmit the transgene in a Mendelian fashion are used for breeding. Expression of the transgene is investigated in following generations by northern blot analysis and by measuring the acid α-glucosidase activity in milk samples. As a result of testing and selective breeding, a line of rabbits was obtained producing recombinant human α-glucosidase (rhAGLU) during lactation.

rhAGLU is extracted from the milk by multistep column chromatography and mixed with infusion fluid for intravenous administration. Safety, tolerance and pharmacokinetic studies were completed in rats and healthy volunteers (phase I) before the start of the phase II clinical study in patients with Pompe disease.

Inclusion and exclusion criteria: The clinical study started with patients having the most severe infantile form of the disease. The inclusion and exclusion criteria are

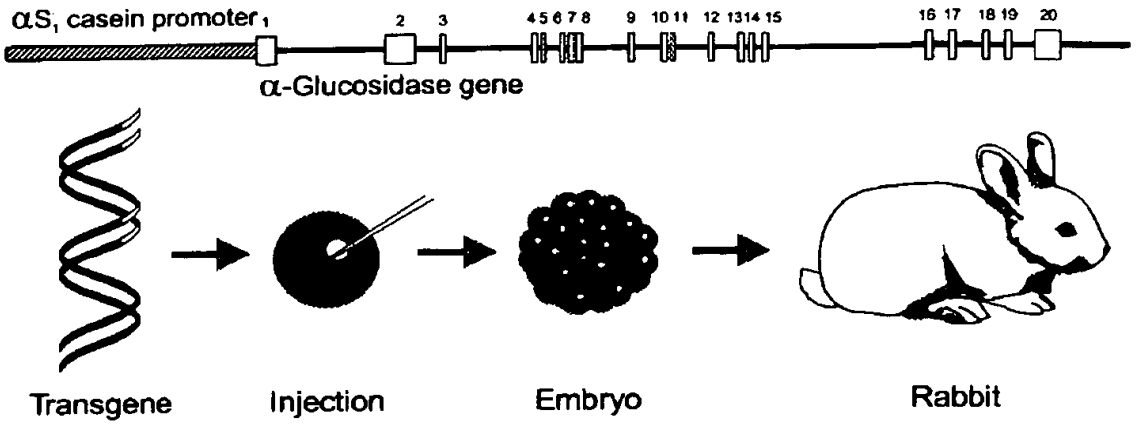

Figure 1 Schematic diagram of the gene construct used to produce recombinant human α-glucosidase in rabbit milk by transgenesis

Table 1 Inclusion/exclusion criteria

Inclusion criteria	*Exclusion criteria*
Symptoms of infantile Pompe disease	Congenital abnormalities
Hypertrophic cardomyopathy	Allergy to food, proteins
Severe α-glucosidase deficiency	Ventilator dependency
Diagnosis confirmed by muscle biopsy	
Age less than 10 months	

listed in Table 1. The clinical presentation of the patient had to be consistent with the most severe infantile form of Pompe disease, including presence of a hypertrophic cardiomyopathy. Cardiac size was determined by ultrasonography. A left ventricular mass index (LVMI) over 68.7 g/m^2 (97.5 centile) was considered to be abnormal (Vogel et al 1991). The acid α-glucosidase activity in fibroblasts and skeletal muscle had to be deficient, and there had to be evidence of lysosomal glycogen storage in muscle upon histological examination. The upper age limit for inclusion of the patients was set at 10 months.

Ventilator dependency was an exclusion criterion, as were allergy to food and medication and congenital abnormalities not consistent with Pompe disease.

Four patients were enrolled (Table 2). They all presented with symptoms before 3 months of age. The final diagnosis was made before 6 months of age, in all cases (14 days, 1 month, 3 months and 6 months, respectively). Two patients were included at the relatively young age of 2.5 and 3 months (patients 1 and 3 in Table 2). They had the best clinical condition at inclusion. A cardiomegaly at birth led to the diagnosis, indicating that the hypertrophic cardiomyopathy already develops during gestation. The youngest of the two was most severely affected. She had signs of cardiac failure

Table 2 Patient details

Patients	*Onset of symptoms*	*Age at diagnosis*	*Age at inclusion*	*Oxygen need*[a]
Patient 1	At birth	1 month	3 months	–
Patient 2	3 months	4 months	7 months	+
Patient 3	At birth	14 days	2.5 months	–
Patient 4	3 months	6 months	8 months	+

[a] During inclusion period

and respiratory distress and was dependent on nasogastric tube feeding from birth. Axial hypotonia, a head lag and slipping through were seen in both cases. The other two patients were included relatively late (7 and 8 months, respectively) in an end stage of the disease. Both patients were oxygen dependent and close to respiratory failure at the time of inclusion. There were signs of cardiac instability. They were hardly able to move their arms, and their legs lay flat on the surface in a frog-like position. Both these patients became respirator dependent: one immediately after inclusion, even before the first α-glucosidase infusion was given; the other 10 weeks after the start of treatment during a bout of pneumonia. The latter patient had a complete atelectasis of the left lung due to extensive cardiomegaly.

Endpoints of the study were exploratory and comprised the gathering of safety and efficacy data. Survival was taken as the primary endpoint.

Safety: This report covers the first 36 weeks of treatment with recombinant human α-glucosidase from rabbit milk (144 infusions in total). Patients over 6.5 kg received 15 mg/kg (patients 1 and 2, in Table 2) and patients under 6.5 kg started with a dose of 20 mg/kg (patients 3 and 4, in Table 2). The dose was increased later to 40 mg/kg for all patients. The enzyme was administered intravenously via a central venous catheter and has been generally well tolerated. Infusion reactions were seen in all four patients, but were well managed by adaptation of the infusion rate. Premedication with antihistamines or corticosteroids was not needed. Changes in blood pressure were never seen. Two patients are treated as outpatients, the other two in hospital.

Uptake of α-glucosidase by muscle: α-Glucosidase activity was assessed in muscle tissue obtained by open biopsy from the quadriceps muscle. All patients had a severe deficiency of α-glucosidase activity in muscle at inclusion ($t = 0$) (Table 3), ranging from 1 to 2% of the normal value (normal value 8–40 nmol/h per mg). Twelve weeks after treatment ($t=1$) with weekly dose of 15 or 20 mg/kg, a second biopsy was taken. A 7–30-fold increase of α-glucosidase activity in muscle was seen, demonstrating that the target tissue was reached. However, the values obtained were still below normal, and the condition of patient 4 became very critical. Therefore, it was decided to increase the dose of all patients to 40 mg/kg weekly. Twelve weeks thereafter

Table 3 Skeletal muscle α-glucosidase activities[a]

Patient	*Activity* (nmol/h per mg)		
	Muscle $t=0$[b]	*Muscle* $t=1$[b]	*Muscle* $t=2$[b]
Patient 1	0.15	4.9	27
Patient 2	0.27	2.7	8
Patient 3	0.2	2.1	13
Patient 4	0.37	2.7	16

[a] Normal α-glucosidase activity 8–40 nmol/h per mg (n=29)
[b] t=0, baseline; t=1, 12 weeks after start of treatment with 15 or 20 mg/kg; t=2, 12 weeks after dose adaptation to 40 mg/kg

($t = 2$) another biopsy was taken. All patients had now acquired normal α-glucosidase activities (Table 3).

Histopathology: At baseline, all patients showed an impressive storage of lysosomal glycogen, as judged by the presence of PAS-positive concentrates in muscle tissue sections (Figure 2A, patient 1). The extent of pathological findings was related to the age of the patient and the severity of symptoms at the time of biopsy. Twelve weeks after dose increase, a significant reduction of PAS-positive material was observed (Figure 2B, patient 1), but the total tissue glycogen content had not changed significantly.

Clinical findings: Normalization of α-glucosidase activity and improvement of tissue morphology was reflected by improvement of clinical condition. The late-included patients had the poorest motor condition at the start of treatment. They were able to lift their arms only briefly, while their legs lay flat on the surface in a frog-like position without any movement. During the 36 weeks of treatment, the patients gained strength in their arms. They learned to play with toys above their head and to transfer objects. Head balance improved slightly. The best effect was seen in the two patients who were included early. At 36 weeks of treatment, the younger of the two could lift her legs freely from the surface and had learned to touch her feet in play. She turned her upper body completely, but was not able to roll over. Her condition is still showing progress. Patient 1 who had the best motor condition at start of treatment, has shown the most remarkable progress. At 9.5 months of age he learned to sit independently without arm support and at 10 months he started to crawl. At 11 months he pulled to a standing position and cruised along furniture. At 12 months, he learned to crawl in a four-point position and to stand with support of one arm.

All patients survived beyond the age of one year, which exceeds the mean age of survival of untreated infantile Pompe patients (Ehlers et al 1962; Willemsen et al 1998). The later-included patients are still dependent on artificial ventilation. The young-included patients, however, did not become dependent on artificial ven-

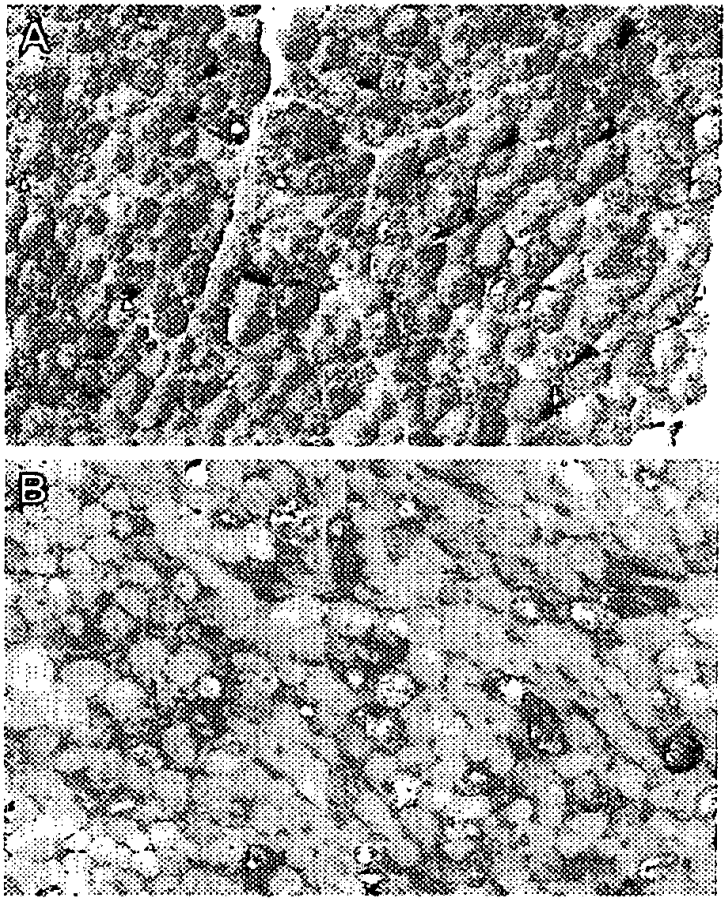

Figure 2 Muscle biopsy (cross-section) from patient 1 obtained before start of treatment with rhAGLU (A) and after 12 weeks of treatment with a dose of 40 mg/kg (B). Sections were stained with PAS to visualize lysosomal glycogen

tilation or oxygen, indicating that the rhAGLU infusions support respiratory muscle function when started early.

The most significant effect was seen on the heart. All patients had a cardiomegaly at the start of treatment, characteristic for infantile Pompe disease. The LVMI exceeded 170 g/m^2 in all, which is well above normal (68.7 g/m^2, 97.5 centile). The cardiac enlargement develops during gestation. The two best-performing patients in the study each had a cardiomegaly at birth. The left ventricular mass index and left ventricular posterior wall thickness decreased significantly after start of treatment with rhAGLU (Van den Hout et al 2000). The largest reduction was seen in patient 4. At 36 weeks of treatment the LVMI had decreased to 30% of the baseline value. Both the atelectasis of the left lung and the signs of cardiac instability had disappeared. The combined effects of rhAGLU were life saving for this patient.

DISCUSSION

This report reviews the first 36 weeks of a study on the safety and efficacy of recombinant human α-glucosidase from rabbit milk in four patients with infantile Pompe disease. The period of treatment is too short for final conclusions to be drawn, but important lessons have been learned. First, evidence was obtained that human enzymes can be produced in the milk of transgenic animals. rhAGLU is one of the first products in this line that are currently being investigated in phase II and III trials. The product is well-tolerated and does not evoke major side-effects. Further, there are several lines of evidence that rhAGLU treatment serves its purpose. Intravenous administration of rhAGLU led over the 36-week period to an increase of α-glucosidase activity in the target tissues. The lysosomal storage of glycogen in muscle seems to decrease, at least when judged by the less intense PAS staining after 36 weeks. However, we have not yet recorded a significant decrease in the overall glycogen content of the muscle at that point. Explanation of this is difficult, as the contribution of lysosomal versus cytoplasmic glycogen to the total glycogen content of the muscle is unknown. Importantly, we did see improved muscle structure in the best performing patient (patient 1). Efficacy of enzyme therapy with rabbit milk rhAGLU is most notable by the decline of LVMI seen in all four patients, along with improved cardiac function. Stabilization of the clinical condition, and for some patients progressive developmental milestones, also point to therapeutic effect. The natural course is one of decline. Patients with severe infantile Pompe disease do not reach major milestone such as sitting and rolling, and usually die around one year of age. Long-term follow up of these children under continuous treatment is necessary to evaluate the true prospects of enzyme therapy for infantile Pompe disease.

Limited as our results are in this early stage, they do show the benefits of enzyme replacement therapy for patients with Pompe disease. It is now time to scale up the effort and make enzyme therapy for Pompe disease a real industrial enterprise. Phase III trials are required and patients with late-onset forms of the disease need to be included. These are the challenges for the near future.

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